Exhibit A-NFLPA-62

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REVIEW

The science behind the quest to determine the age of bruises—a review of the English language literature

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Abstract Bruises are common injuries that can have medicolegal significance. There are those that maintain it is not possible to estimate the age of bruises. However, appreciation of the biological processes related to the resolution of a bruise suggests that these may provide information regarding the age of a bruise. Potential methods for determining the age of bruises—visual observation, colorimetry, spectrophotometry and histology—are reviewed. The observation of yellow (not orange or brown) indicates a bruise is not recent, but the abilities of visual observation are limited by the physiology of the human eye. Analysis of spectrophotometric data may provide more useful and objective information. Histological examination may be appropriate only in the postmortem situation. The lack of published information limits this as a tool for estimating the age of bruises. It is not known how the wide range of factors that can influence bruise formation and resolution could affect estimation of bruise age.

Keywords Bruise · Skin · Human · Time factors · Color perception · Colorimetry · Spectrophotometry · Histology · Pathology · Review · Forensic

The word bruise is derived from the old English word *brysan*, which means to crush [1]. For a bruise to occur, three criteria have to be satisfied. First, the skin has to be stretched or crushed sufficiently to cause tearing of blood vessels within it or in the underlying fat, but without resulting in loss of integrity of the surface [2–4]. In order for this to occur, the skin must be deformed by a blunt instrument, otherwise the skin will be cut before vessels tear. If the elastic limit of the surface of the skin being deformed is exceeded, a laceration will form (the skin is split). Conversely, the degree of trauma may be minimal and not noticed if the vessels are fragile, for example in the elderly [5]. Second, after the vessels in the skin are damaged there has to be sufficient pressure of blood to cause escape of red blood cells from vessels into the tissue. Third, the escaped blood must be sufficiently near the surface of the skin to be visible. This will vary with the optical properties of the skin. A bruise inflicted during life might not be visible due to the opacity of skin [6–8], but it will be revealed postmortem by skin reflection [9, 10]. Bruises may not be inconsequential injuries as they can result in death if extensive [11, 12].

The early appearance of a bruise is dependent on two factors: escape of hemoglobin-containing erythrocytes from blood vessels into the tissue and the depth of the blood within the skin [13–15]. Over time, the appearance depends on diffusion of hemoglobin through the tissues and removal of hemoglobin by the inflammatory response [1].

Hemoglobin has the role of transporting oxygen from the lungs to the tissues of the body. It is composed of a porphyrin ring around an iron atom [16]. The spectrum of deoxyhemoglobin has a sharp peak at 430 nm and broad peak around 555 nm. It appears slightly darker than the bright red of oxyhemoglobin, which has peaks around 415 nm, 540 nm and 575 nm [17–19]. The presence of hemoglobin near the surface of the skin will appear red, but release of blood deeper into the tissue is said to appear blue, an effect that is attributed to Rayleigh scattering, absorption coefficients of skin and interpretation by the visual system [13, 15, 18, 20]. A similar effect is seen with melanin, which appears as a yellow pigment when superficial, but can appear blue when deep [20, 21].

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The release of blood into the tissue will evoke an inflammatory reaction [22] and this response may be accentuated by tissue damage due to blunt trauma [23, 24]. Neutrophils are the first cells to arrive, but these probably cannot degrade hemoglobin [4, 25]. Macrophages can phagocytose erythrocytes [26] and they contain heme oxygenase that enables the first step of hemoglobin breakdown. Hemoglobin is split into biliverdin by heme oxygenase [27]. This reaction is energy-dependent, requires oxygen and results in the release of carbon monoxide and the iron atom. Biliverdin is a green pigment and it is rapidly changed to bilirubin, a yellow pigment, by the enzyme biliverdin reductase [28, 29]. It is likely that the free iron is locally bound to ferritin [27] and that hemosiderin is a polymer of ferritin [30, 31]. It also appears that bilirubin can accumulate to form local yellow crystals that have been referred to as hematoidin, [32] which may dissolve in tissue processing [33].

Heme oxygenase has been recognized as having a potential role in modulation of the inflammatory response [25]. Two isoforms have been characterized; inducible heme oxygenase -1 and constitutively expressed heme oxygenase -2, which is found in a wide range of tissues [27, 34]. The form within macrophages is heme oxygenase -1 and it is usually present at low levels [35]. Its levels rise following phagocytosis of erythrocytes [25, 26, 33] or exposure to hemoglobin [28] and it may be detected by immunohistochemistry as early as 3 h following the release of blood into the tissues in humans [36]. Heme oxygenase is also present in an inducible form in fibroblasts (present in the skin) [37], but the role of fibroblasts in clearing of hemoglobin in a bruise has not been established [37].

Thus, the initial appearance of a bruise is due to the ability to perceive blood that has been released within the skin (for these purposes, the earliest reaction to trauma—the wheal and flare response [38, 39]—will be ignored). A bruise may become established as early as 15–20 min after injury (in a porcine model, struck by a paintball) [40]. It is written that the early color depends on the depth of the blood within the skin [15, 18, 41]. Colors that may be reported as red, blue, purple, black or green are no guide to the age of a bruise [42]. It may be that some of these colors are perceived as a result of the effect of contrast on the human eye–brain visual system rather than due to their actual presence [18, 43]; this could occur because color is a perception, not a property of the skin [44]. Changes in the colors seen in a bruise will occur due to shifting of the position of blood relative to the skin surface [1] and may be augmented by the released hemoglobin changing from oxyhemoglobin to deoxyhemoglobin [45]. In addition, it has been recently suggested that production of carbon monoxide during the catabolism of hemoglobin [37] could allow the local formation of carboxyhemoglobin, which could make bruises appear a brighter red [46].

The production of bilirubin and hemosiderin at the site of a bruise requires time for macrophage recruitment, inducement of heme oxygenase and catabolism of hemoglobin [35]. The development of yellow color in bruises has been attributed to the local production of bilirubin, [28, 47] which can be justified, as raised serum bilirubin levels correlate roughly with the yellowness of the skin in jaundiced neonates [48]. Observation of a bruise, either directly or from photographs, has been the traditional method by which the age of a bruise may be estimated. Many old forensic textbooks [49–54] and even more recent publications [55] include guides to determining the age of a bruise based on its color. However, consideration of the biological processes occurring in a bruise supports the conclusion that only the appearance of the color yellow would provide any information regarding the age of a bruise when using observation alone [42]. If a bruise is directly accessible it could be suggested that recording factors such as tenderness and swelling might assist with determining the age of the injury [56], but there is no evidence that this would be the case.

Thus, the perception of any color such as 'blue', 'green', 'purple', 'black', 'orange', 'brown' or 'red' indicates nothing about the age of a bruise. Statements such as 'a bruise that is blue is recent' cannot be substantiated—it could well equally be old. Similarly, 'a fresh bruise will be red' is not justifiable, as an old bruise may be red: [42, 57, 58] consider 'senile purpura', which tends to retain its red color. Some have stated that the presence of green color in a bruise reflects the presence of biliverdin; indeed, biliverdin may be present in some forms of jaundice, where it will impart a green tinge to the skin [59]. However, there is no evidence to support that biliverdin will accumulate in bruises in humans [60–62], but it may develop in some animals [60, 63, 64]. Mathematical modelling of the optical properties of deep blood indicates that it may appear as blue or turquoise color [18]. Green could be perceived from a mixture [65] of this with yellow from skin pigmentation (see below).

If yellow is seen in a bruise, then that bruise is not recent [66] (provided it has not been inflicted on the site of a pre-existing older injury that was already showing yellow color). There are a number of important points to this statement.

'Yellow' means bright yellow, not 'orange or 'brown'—to see an example of true yellow use any painting or image processing program with the color values red 240, green 240, blue 0. This author has seen the colors orange and brown in very early bruises, most notably in infants. However, orange and brown may be perceived due to the presence of methemoglobin. Methemoglobin has a brown color [67]. It forms when the iron molecule in hemoglobin is oxidized from 2^+ (its normal state) to 3^+.

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Oxyhemoglobin will oxidize into methemoglobin at a rate of up to 3% per day, [68] but this may be accelerated in conditions of low oxygen tension [69] (which would be expected within a bruise) and neutrophils (that might be present in an early bruise) can oxidize hemoglobin to produce methemoglobin without phagocytosing erythrocytes [70]. Normally, methemoglobin is recycled back to hemoglobin, but the reaction is energy-dependent [69, 71]. Infants are vulnerable to the production of methemoglobin [72]. Methemoglobin can be detected in bruises, [45] which could explain the observation of orange or brown color in early bruises. However, this requires experimental verification.

The observer has to be able to perceive yellow. Tests have demonstrated a wide variation in the threshold for yellow perception in the population and sensitivity for yellow deceases with age [73, 74]. Others have also shown that there are individual differences in color perception and reporting [75, 76]. In addition to a physiological inability to perceive yellow, an observer may not see yellow in a bruise because it is masked by dominance of color by the blood within the bruise or due to pigmentation of the skin [77]. As there are yellow pigments in the skin [78], mainly melanins [79–81] and carotenes [13, 75, 82, 83], the yellow color in a bruise may become masked [84].

It has not been rigorously established when yellow appears in a bruise. The published study stated that yellow was not seen in a bruise that was less than 18 h old, and that remains true for this author [42]. That yellow can be seen in such early bruises was a surprising observation at the time. However, this was a minority observation, with most bruises taking at least 24 and nearer 48–72 h to show any yellow color. This is consistent with findings by others [45]. The published observation should not be interpreted as an expectation to see yellow in bruises that are 18 h old, as most will have none. Also, some bruises will never show any yellow color. In hindsight, the study lacked rigour as it was based solely on the observations of the author, with no formal testing of interobserver or intraobserver variation. Nonetheless, the observer was 'blinded' to the true age of the bruises when the observations were made. At least others have recorded similar observations [4, 57]. Yellow in a bruise is contingent upon the local production of breakdown products of hemoglobin (bilirubin and hemosiderin). When the presence of these substances is perceived, such a perception may be more dependent on the qualities of the human visual system than their actual levels. Digital image analysis has been used for the study of burns and scars to provide objectivity of color measurement [85–87]. This author did attempt a re-assessment of the original photographs [42] using a digital image analysis system in order to increase the objectivity of the study. However, many of the photographs could not be analyzed due to uneven lighting. Although overall the results were in agreement with the original study, there were insufficient data points to be conclusive. This emphasizes the need for rigorous control of lighting and conditions when photographing bruises [85]. The colors in the end image will be affected by glare [19] and illumination [88] as well as other factors, such as the film and processing. This author recommends including a standard color scale in all photographs to attempt to minimize color distortions that could affect interpretation of a bruise. Other authors have attempted to increase the objectivity of the observation of bruises using colorimetry, which will be discussed below.

Published work on estimating the age of bruises was based on observation of adults, with only 7 subjects being aged under 16 years. There was a significant difference in the average time to first appearance of yellow color between subjects aged up to 65 years and those aged 65 years and over [42]. It has been reported that bruises in younger animals heal faster [89] and that macrophage function is impaired in the elderly [90, 91]. Consequently, there may be a difference in bruise resolution between human adults and children. The lack of literature regarding estimating the age of bruises in infants and children has been noted [92] and it has been suggested that studies be performed specifi cally on children [56, 92, 93]. However, it may be that the pattern of bruising and correlation to the history are of more importance than the age of bruises in distinguishing accidental injury from abuse [92, 94–98].

As a further point, when researching bruises it is important to be sure of the age of the bruise *from the time of injury*. A subject may state that his bruise is a day old, but by asking *how* the bruise occurred it will often become apparent that the bruise was *noticed* a day ago, but the actual time from injury is unknown. From experience, this phenomenon is common. A lack of certainty of the cause of bruises could limit the interpretation of findings [58].

Finally, to reiterate, a bruise may never become yellow. Therefore, when using visual assessment alone nothing can be determined regarding the age of a bruise that does not show yellow color (a bruise that does not appear yellow can be of any age) [42]. However, it may be possible to determine something useful about the age of a bruise that is not yellow using equipment that is more sophisticated than the human eye.

The normal eye has three color receptors (cones) that respond to regions of the visible spectrum termed red (558 nm), green (530 nm) and blue (426 nm) [99]. However, the spectral responses of the cones of the eye are not highly specific and their sensitivities overlap. Nonetheless, for a person with normal (trichromatic) color vision, any color can be reproduced by light of the three primary colors (red, green and blue) in the appropriate ratios [44, 99]—which is the basis behind color television [100]. Yellow

color is perceived when the red and green cones are stimulated equally and there is no blue stimulus, with the processing of the signals being performed in the optic cortex [101]. The threshold for yellow perception varies between observers [74]; it is dependent on factors including the incident light [102], opacity of the lens of the eye and the spectral response of the cones [103–106]. Colorimetry may provide an objective means for the assessment of color in a bruise, avoiding individual variations in color interpretation [44, 75, 107, 108].

A colorimeter uses a (white) light source and three receptors tuned to the red, green and blue regions of the spectrum. The output from the receptors can be processed (using electronics within the device) into color data [4, 78]. This can be presented as red, green and blue (RGB) values, but visible color can be represented by any appropriately transformed set of 3 independent variables, which is the basis behind other color systems such as hue, saturation and intensity (HSI) and L*a*b* [81]. The L*a*b* color model was devised in 1976 by the Commission Internationale d'Eclairage (CIE) to closely represent the perceptual range of normal human vision and to allow measurement of differences in color [100, 102]. The L* value corresponds to luminosity (brightness), a* is the green-red axis and b* is the blue–yellow axis (with yellow having a positive value) [109, 110]. It would be expected that this color model would be advantageous for the study of color in bruises, particularly for the measurement of yellow (although other measurements specifically for yellow exist [111]). Use of colorimetry does permit objective analysis of color data and can be used to quantify skin color [20, 41, 100, 110, 112]. However, testing using a large set of bruises of known age (a study based at this department of 233 bruises from 149 subjects) revealed that the background color of the skin is a significant confounding variable when using colorimetry to estimate the age of bruises. This finding may be a result of limitations of the CIEL*a*b* system [19] and the use of other color models might provide better results [111, 113]. However, it is likely that any system that studies bruises needs to use more than just three color data points [114], even when supplemented by taking measurements on two separate occasions [108].

Spectrophotometry provides the ability to measure multiple points within the visual spectrum [81] and the intensity can be measured at 1 nm intervals over the entire visible range red (700 nm) to blue (400 nm) [19, 80, 115]. Information that may be of use in determining the age of a bruise can be extracted from the spectrophotometric data [116, 117]. For example, the proportion of oxygenated hemoglobin within skin can be estimated by spectrophotometric measurements [45, 118]. Blood that is released into a bruise is initially predominantly oxyhemoglobin, but it rapidly becomes deoxyhemoglobin, potentially providing information regarding the early age of a bruise [45]. Calculation of the first derivative is a more advanced mathematical transformation that can be performed on spectrophotometric data. The first derivative provides a value for the rate of change of absorption as the wavelength changes. This can be utilized to measure levels of bilirubin in a mixture with hemoglobin [119, 120]. Over the range 470–510 nm the absorption spectrum of hemoglobin is almost flat, but the absorption of bilirubin is decreasing from its peak around 460 nm [17, 119] and hemosiderin has a sloping absorption curve [121]. Therefore, the value of the first derivative around 480–490 nm corresponds to the presence of end degradation products of hemoglobin. This value may be of use in estimating the age of a bruise [122]. Unlike the measurement of yellow color, it was not confounded by skin pigmentation in a study conducted at this department using spectrophotometry to estimate bruise age (although there were no negroid subjects). Initial experiments using spectrophotometry with advanced mathematical modelling have indicated promising results for determining the age of bruises [123].

Hyperspectral imaging (also termed chemical imaging) has the potential to provide spectrophotometric information for large areas of the body to include whole bruises [124]. A hyperspectral imaging device can be regarded as a camera with a spectrophotometer as the imaging device [125, 126]. One design uses a tuneable filter between the lens and the electronic imaging plate (similar to the types used in digital cameras) to capture a full spectrum for each pixel (element) of the image. The location and extent of hemorrhage [127] as well as the presence of bilirubin in bruises can be measured using hyperspectral imaging, which has the advantage of being able to select an area of interest for analysis from within the imaged area [128].

Further information may be gained by using infrared spectrophotometry systems, because infrared has a greater penetration into the skin [129–131]. It may be possible to gain information regarding water content [132] to assess oedema and measure hemoglobin [133, 134].

At the other end of the spectrum, bruises have been studied using ultraviolet (including Wood's) light. While this may accentuate some injuries [135–137], it is unlikely that it will assist with determining the age of bruises [138].

The penetration in skin by light is limited by scattering and absorption, with blue light penetrating around 100 μm and red light penetrating at 500–750 μm [19, 130], limiting the use of observation and reflective techniques. Transillumination of the skin by diaphanoscopy [139, 140] might enable analysis of bruises deep in the skin, but sampling a bruised area of skin by excision biopsy would be expected to yield more information for estimating the age of a bruise. Unfortunately, there is a lack of published histological studies of bruises of known age. Probably one of the best was

conducted on bruises of sheep. Using Bayesian analysis of histological features it was concluded that bruises could be only aged as 0–20 h or 24–72 h with any accuracy [141]. Other work in lambs and calves indicated that neutrophils were numerous at 8 h with blood and fibrin; macrophages and neutrophils were present in approximately equal numbers by 24 h and macrophages predominated by 48 h [142]. However, data from animal experiments may not be applicable to humans [33]. Neutrophils are not present in normal skin, thus the finding of any would be regarded as signifi cant [143]. Emigration of neutrophils within minutes has been reported in blunt trauma skin wounds in humans [144]. In lacerations and incised wounds of the skin, neutrophils (a minimum of 10 outside the area of bleeding) have been reported in 20–30 min in humans [145]. A neutrophil infiltrate has been recorded as early as 5 min [146] and 10 min in studies of human closed head injuries [147]. Immunohistochemically, neutrophils were found in the perivascular region after 20–30 min and within the stromal tissue by 30–40 min in human skin wounds [148]. Nonetheless, traditionally, neutrophils would not be expected to be numerous until several hours have elapsed from the time of injury [149, 150]. Macrophages are present in normal skin [143] making it more difficult to determine when an infiltration has commenced. They are increased from around 12 h in traumatic lesions of the human brain [146, 151]. In human skin wounds, macrophages have been reported from 3 [145] or 7 [152] hours, with a peak around 1–2 days, [152] outnumbering neutrophils from 20 h [145]. Erythrophagocytosis appears from 12 h in brain injuries [146, 152] to 3 days in skin wounds [145]. Marked variation of the inflammatory infiltrate in burn wounds of the same age occurs between subjects [143] and the same could occur in bruises—particularly if there is devitalization of tissue. The presence of hemosiderin can be demonstrated using Perls' stain [153] at 3 days in human skin wounds, [145, 154] but earlier in mice [32] and other animals [142]. Hemosiderin is not seen before 48 h in brain injuries [155] and it takes at least 4 days for stainable hemosiderin to form [146, 156, 157]. How long hemosiderin may persist in the skin is unclear. It has been found to remain for months in the retina of monkeys following retinal hemorrhage [158] and has been found in a biopsy taken 12 days after an episode of pulmonary hemorrhage in a 13–week-old girl [159]. This raises the possibility that hemosiderin seen in a bruise could relate to an earlier traumatic event.

Immunohistochemical study of proteins that are part of the wound healing process [160–168] might provide information regarding the age of bruises. This may be supplemented by the use of molecular techniques [148, 169, 170]. However, care must be taken with regard to the selection of control material [165, 171]; artefacts may occur [166, 172] and findings must be verified on postmortem tissue [173].

Challenges facing any method that may be used to estimate the age of bruises are the range of variables that affect the formation of a bruise and biological differences between bruised subjects. The resolution process of a bruise may also be influenced by some factors.

The appearance of a bruise will depend on the force used. It has been found that a slow-moving but heavy mass is less effective at causing a bruise than a fast-moving object [89], with a heavy mass causing deep, rather than skin, bruising [39, 40]. An impacting object with squared edges is better at forming bruises in the skin than a rounded one [39]. A bruise is more likely to occur over an area supported by bone, but laxity of the tissue is also important: consequently it is harder to bruise the skin of the abdomen, which is unsupported, or the palm of the hand, which is collagenous, than the face, which is supported by bone, lax and vascular—a further factor important in the genesis of bruises [2, 4, 8]. It is often stated that some people 'bruise easily'. This may be explained by factors such as fragility of the skin, blood clotting abnormalities [174, 175], deficiency states such as scurvy [176] and drugs including isoretinin [177] or steroids [178]. In some there may be a role for prostacyclins [179]. In other cases, the cause could be in sight, but not apparent [180]. However, females of child-bearing age can be affected without apparent explanation [181]. Local cooling can reduce the amount of bruising [182]. The resolution of bruises may be altered by local treatment [183, 184] and repeated bruises on the same site heal faster [63]. Anecdotally, bruises resolve faster if the area is massaged or rubbed. Exposure to sunlight might speed the removal of bilirubin by converting it to lumirubin and other isomers that are more soluble in water [127, 185, 186]. Whether these variables will affect the estimation of the age of bruises is unknown.

In the postmortem situation, the issue may not be the actual age of the bruise, but if it occurred before or after death. There is the potential for a bruise to develop after death [187–189], particularly if there is passive pressure acting on the blood [190–192], for example if the injured area is dependent [193, 194]. In contrast, if pressure is maintained on an area until, and even after, death has occurred, then the bruising may not develop [195]. The distinction of postmortem from antemortem bruising may not be easily made as blood can extravasate postmortem to lodge in the tissue and not wash off, resembling antemortem bruising [190, 196–198]. Presumably, the formation of bruises after death would be favored if blood is in a fluid state [199–201]. It is conceivable that bruises could be masked by postmortem hypostasis, particularly on the back when hypostasis is well developed [4] and then be revealed when the blood has been released from the tissue [38]. In a decomposed body, bruises may become undetectable due to discoloration of the skin [202]. Bruises can be mimicked by

areas of skin discoloration or diffusion of hemoglobin through the blood vessel wall [203]. It has been stated that immunohistochemical demonstration of glycophorin A (a protein in the erythrocyte membrane that is resistant to decomposition) in the interstitium is indicative of extravasation of blood having occurred, [203, 204] but this does not indicate that the injury occurred antemortem. Observation of changes in elastic fibres is also not discriminatory [205] and alteration of collagen fibres can occur due to postmortem trauma [206]. Furthermore, hemocoagulation and fibrin formation has been described postmortem [207, 208]. Notwithstanding this, the finding of a local vital reaction may be the only sign that a bruise occurred antemortem [149, 209–211]. An established infiltrate of neutrophils would be expected to imply that a bruise occurred during life, as neutrophils are not present in normal skin [143]; however, it has been observed that neutrophil emigration can occur after death in response to cytokines [212]—at least in mice [213]. Also, care must be taken to exclude inflammatory cells that are present due to passive 'drift' [145]. Histochemical [149, 207, 209, 211, 214, 215] and immunohistochemical [165, 166, 168, 216] methods may prove to be helpful in distinguishing antemortem from postmortem wounds. An infiltrate of macrophages would be evidence that the injury occurred during life. The lack of a vital reaction does not imply that the injury occurred postmortem [197].

In the future, advanced technologies such as proteomics [217] or magnetic resonance imaging (MRI) [218, 219] may assist in determining the age of bruises. At present, the best that can be achieved from visual inspection is to state that a bruise that is yellow is not recent. If histological examination can be performed, stainable hemosiderin implies that it is at least a couple of days old. However, any statements must be made with caution, as uncertainties exist (see above). Rather than attempting to assign an age to a bruise, it may be more practical to ask when it is thought the bruise was inflicted and then to convey if the appearances are consistent or not consistent [220]. There may be cases when the only correct answer is that the age of bruise cannot be determined. This author agrees with those that believe that it is not possible to precisely determine the age of a bruise [221, 222]. However, research rather than nihilism is required to objectively determine the limits of confidence that can be achieved with regards to estimating the age of a bruise such that the judiciary can be correctly informed.

Educational message

1. Yellow (not orange or brown) is the only color that may provide any information regarding the age of a bruise.
2. Yellow may be apparent in a bruise from 18 h after its infliction.
3. Nothing can be determined regarding the age of a bruise that has no yellow when an observer is restricted to visual inspection alone.
4. Other techniques, such as spectrophotometry, may provide more information regarding the age of a bruise.
5. The published literature indicates that stainable iron will not be seen in a bruise before 3 days.
6. There are many variables that could potentially affect the ability to estimate the age of a bruise.

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